fluorescent protein to report target sequence expression and enhanced green fluorescent protein for microRNA expression. Using this assay, we demonstrated a functional target for miR-212 in the 3′ untranslated region of $K_{ir}2.1$. Red/green fluorescence intensity ratio was significantly lower in miR-212-expressing HEK293 cells compared to non-targeting control (miR-212 0.72 ± 0.024 (mean ± sem), n = 550; control 1.21 ± 0.025, n = 731; $p<.001$, log transformed data). The effect of miR-212 was attenuated by mutating the predicted target site (% inhibition 58.0 ± 14.51, n = 3 wild-type; 22.7 ± 1.25, n = 3 mutant). Expression of miR-212 downregulated endogenous $K_{ir}2.1$ protein in HeLa cells, as shown by quantitative western blot of membrane extracts (band intensity *vs* Na^+/K^+-ATPase loading control: miR-212 0.0647 ± 0.0047; non-targeting control 0.0895 ± 0.0045; n=3, $p<.05$). Endogenous inward rectifier K^+ current in HeLa cells was isolated by extracellular application of 100 μM Ba^{2+} during whole-cell patch-clamp recording. Ba^{2+}-sensitive current density was significantly smaller in miR-212-transfected (n=13) *vs* control-transfected cells (n=8); $p<.01$. In conclusion, downregulation of inwardly rectifying K^+ current and $K_{ir}2.1$ expression in heart failure and alcoholic cerebrovascular dysfunction may be functionally linked to upregulation of miR-212.

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Dissecting Gating Rules of GIRK Channels: Role of PIP2 in Ethanol-Dependent Activation

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G protein-gated inwardly rectifying potassium (GIRK) channels are implicated in alcohol abuse and addiction. We discovered a discrete alcohol-binding pocket in the channel mediating ethanol-dependent activation. Here, we investigated the role of G proteins and PIP_2 in ethanol-dependent gating. We engineered GIRK2 with single, modifiable cysteine at L257 in alcohol pocket and found that alcohol-like methanthiosulfonate (MTS) reagents activate GIRK2-L257C, similar to ethanol-dependent activation. We assessed role of G proteins in alcohol activation by either increasing levels of Gβγ (+Gβ1γ2) or decreasing Gβγ through chelation with membrane bound Phosducin (+mPhos). Neither Gβ1γ2 nor mPhos altered the rate of MTS-HE-mediated GIRK2-L257C activation. For comparison, we examined GIRK2-L344C, a key site for Gβγ activation that is inhibited by MTS modification. In contrast to L257C, rate of MTS modification showed a clear dependence on Gβγ levels. These results suggest that alcohol activation of GIRK channel is independent of G proteins. To investigate the role of PIP_2, we used voltage-sensitive phosphatase DR-VSP to transiently deplete PIP_2 in the membrane. Activation of DR-VSP completely reversed MTS-HE activated current of GIRK2-L257C. Furthermore, MTS-HE treatment significantly slowed the rate of GIRK2-L257C current inhibition following DR-VSP activation, suggesting an increase in apparent affinity for PIP_2 and GIRK2-L257C channels modified by MTS-HE. Lastly, we examined the role of PIP_2 on alcohol-dependent activation of wild-type GIRK2. Addition of propanol significantly slowed the rate of wild-type GIRK2 current inhibition following PIP_2 depletion. Taken together, these data demonstrate that alcohol-dependent activation of GIRK involves an increase in apparent affinity for PIP_2, with little influence of Gβγ subunits. The fundamental dichotomy between alcohol and Gβγ arises from distinct gating mechanisms converging on PIP_2-dependent opening, revealing novel pathways for antagonizing alcohol's effects on ion channels.

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Unique PIP2 Sensitivity at a Putative PKC Site in GIRK2 (Kir3.2)

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G-protein activated inward rectifier potassium channels (GIRKs) exhibit sensitivity to a diverse range of modulators, including G-proteins, sodium, PIP_2, and phosphorylation by PKA and PKC. The residue Ser-196 in the GIRK2 subunit is implicated in PKC sensitivity in the homologous GIRK1 and GIRK4 subunits. It is located distal to the helix bundle crossing, and is situated to interact with both Phe-192 of the bundle crossing and Thr-317 of the G-loop gate. In the background of the highly active homomeric GIRK2 mutant E152D, we mutated Ser-196 to Ala and tested its PIP_2 dependence, using the voltage-sensitive PIP phosphatase Ci-VSP. The S196A mutant was inhibited normally by activation of Ci-VSP, but upon recovery it displayed a unique behavior. Instead of a monophasic recovery, the S196A mutant exhibited a characteristic inhibition following recovery, which was not observed in the homomeric mutant alone. In addition, the S196A mutant current recovery depended on the initial level of PIP_2 depletion. Mutant channels S196E and S196Q did not reproduce the unique pattern of S196A. Using the G_q-coupled hM1 assay, we tested the muscarinic sensitivity of S196A vs. homomeric mutant. While the homomeric mutant was inhibited normally, the S196A channel did not show appreciable inhibition. Taken together, these results indicate that the S196A mutant exhibits unique PIP_2 sensitivity. Given the critical location of S196 to the channel gates we are pursuing the structural mechanism that could explain the unique behavior of the Ala mutant.

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HDAC Inhibitors Affect Sulfonylurea Receptor Subunit MRNA Expression in Atrial-Derived Hl-1 Cells but not Pancreatic Beta Cell-Derived MIN6 Cells

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K_{ATP} channels are expressed in many types of excitable cells where they typically act as key sensors of cell metabolism. All K_{ATP} channels share the same architecture—a K^+ channel pore (Kir6.1 or Kir6.2) combines with a sulfonylurea receptor (SUR1, SUR2A or SUR2B) to form a functional channel. Importantly, K_{ATP} channels composition is tissue specific. SUR1 and Kir6.2 make up the channel in atrial cardiomyocytes and pancreatic beta cell, while SUR2A combines with Kir6.2 to form ventricular myocyte K_{ATP}. Tissue specific heterogeneity appears to be driven principally by differential subunit transcription, but the mechanisms that determine when and where specific K_{ATP} channels are expressed are poorly understood. In this study, we have employed both cardiac- (HL-1) and pancreatic beta cell- (MIN6) derived cell lines to explore the mechanisms that control SURx gene expression. In both HL-1 and MIN6 cells we find that SUR1 expression is significantly greater than SUR2. When cells are treated for 72 hours with trichostatin A (a general inhibitor of histone deacetylases or HDACs), there is a significant increase in SUR2 subunit expression in HL-1 cells, but no apparent change in SUR2 expression in MIN6 cells. This result indicates that in the absence of HDAC activity, the transcriptional machinery to drive SUR2 gene expression is available in HL-1, but not in MIN6 cells. From this data, we conclude that both the SURx subunit transcriptional profile and the mechanisms that determine that profile are tissue specific.

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Lessons from KATP Channels with Diabetogenic Mutations in Sulfonylurea Receptor 1 (SUR1)

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Numerous mutations have been identified in SUR1 (ABCC8) subunit of the neuroendocrine type KATP channel in subjects with neonatal diabetes, neonatal diabetes plus epilepsy and/or other neurological features, maturity-onset diabetes of young, and later-onset diabetes. Patch-clamping, single-channel kinetics analysis, affinity photolabeling and molecular modeling were used to clarify how diabetogenic mutations in different parts of SUR1 affect open probability and sulfonylurea inhibition of SUR1/Kir6.2 KATP channel. Essentially all tested diabetogenic mutations in the canonical TMD1-NBD1-TMD2-NBD2 ABC exporter core of SUR1 hyperactivated KATP by stabilizing the stimulatory Mg-nucleotide bound (outward facing) state of SUR1 without affecting the intrinsic gating of KATP channel or its sensitivity to inhibitory nucleotides. Hyperstimulated mutant channels showed attenuated sulfonylurea inhibition in the presence, but not the absence, of stimulatory MgATP/ADP, indicating that KATP with SUR1 in the inward facing state has the lowest Kd for sulfonylureas. Diabetogenic mutations in the non-canonical TMD0-L0 part of SUR1 hyperactivated KATP by destabilizing its long-lived closed state with the highest affinity to inhibitory ATP or by strengthening the functional coupling between the MgATP/ADP-bound SUR1 core and the active (burst) state of the Kir pore. Mutations destabilizing the long-lived closed state compromised sulfonylurea inhibition of KATP in the absence of nucleotides but not the drug-induced release of stimulatory nucleotides. The findings support the mechanistic model (FEBS Letters, 585:3555-9) in which the TMD0-L0 module couples the SUR1 core with the KATP pore, define the most common ABCC8-associated mechanisms of KATP hyperactivity, and largely explain why the majority of diabetic subjects with mutant SUR1 require body-weight normalized doses of sulfonylureas exceeding those recommended by the FDA for treatment of common type 2 diabetes.

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A Single Point Mutation in the Distal C-Terminal of the Pore Forming Kir6.1 Subunit Modifies ATP-Sensitive Potassium (K_{ATP}) Channel Regulation

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K_{ATP} channels are hetero-octamers composed of two subunits; a pore forming Kir6 and a sulphonylurea receptor (SUR). SUR subunits regulate K_{ATP} channel gating allosterically, in response to nucleotides and pharmacological agents.

We have previously shown that residue D323 of Kir6.2 is central to interaction with the nucleotide-binding fold 2 of SUR2A (Rubaiy et al, Biophysical J. 2011: 100; P432a). The aim of this study was to assess whether E332, the corresponding residue in Kir6.1, plays an equally important role in Kir6.1/SUR2A complexes. Wild type Kir6.1/SUR2A channels expressed in HEK293 cells required activation by potassium channel opener pinacidil (EC_{50} = 43.90 ± 1.28 μM), in the presence of UDP (10 mM) before passing current. Introduction of the single point mutation E332K into full length Kir6.1 caused constitutive opening of Kir6.1/SUR2A channels in the absence of pinacidil. Reinstatement of putative inter-subunit salt bridges by expressing Kir6.1-E332K with charge reversal mutants SUR2A-Q1336E or SUR2A-K1322D failed to restore regulated opening. Channels containing the Kir6.1E332K mutant were also insensitive to block by high concentrations of glibenclamide (100 μM). However, co-expression of Kir6.1-E332K with SUR2A-K1322D restored glibenclamide sensitivity to wild type levels ((IC_{50} = 9.12 ± 1.12 nM), $p < 0.15$ versus wild type Kir6.1/SUR2A channel (IC_{50} = 6.14 ± 1.13 nM). Together, these data suggest a key functional role for inter-subunit salt bridges involving Kir6.1-E332K. Constitutive channel opening on mutation of this residue suggests that Kir6.1E332 and salt bridge(s) formed between it and the SUR subunit are crucial for stabilizing closed states of Kir6.1-containing K_{ATP} channels.

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A Cytoplasmic Inter-Subunit Salt Bridge, Kir6.1R347/SUR2AE1318, Contributes to Allosteric Information Transmission in Kir6.14/SUR2A4 Channel Complexes

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K_{ATP} channels are hetero-octamers composed of pore forming Kir6 (Kir6.1 or Kir6.2) and a regulatory sulphonylurea receptor (SUR1, SUR2A or SUR2B) subunits. Previous studies have revealed a salt bridge between Kir6.2K338 and SUR2AE1318 that is involved in inter-subunit transmission of allosteric information (Rubaiy et al, Biophysical J. 2011: 100; P432a). The aim of this study was to assess whether Kir6.1R347, which corresponds to Kir6.2K338, plays a similar role in Kir6.1-containing channels.

Whole-cell patch clamp recording was employed to assess channel sensitivity to pinacidil and glibenclamide following mutation of single residues or reinstatement of the proposed salt bridge by paired charge reversals in full length Kir6.1/SUR2A channel subunits after heterologous expression in HEK-293 cells. A single point mutation, Kir6.1R347E expressed with wild type SUR2A increased significantly ($p < 0.006$) the sensitivity to activation by pinacidil of Kir6.1R347E/SUR2AWT channels (EC_{50} = 0.71 ± 1.21 μM) versus wild type Kir6.1/SUR2A channels (EC_{50} = 43.90 ± 1.28 μM). Reinstatement of the cytoplasmic electrostatic interaction in the Kir6.1R347E/SUR2AE1318R subunit combination reversed the sensitivity to pinacidil to near wild type (EC_{50} = 23.5 ± 1.3 μM, $p < 0.028$). Furthermore, glibenclamide sensitivity was reduced significantly in the Kir6.1R374E/SUR2AWT channel (IC_{50} = 241 ± 1.09 nM, $p < 0.015$) and restored in Kir6.1R347E/SUR2AE1318R (IC_{50} = 13.75 ± 1.11 nM, $p < 0.080$) versus wild type Kir6.1/SUR2A channel (IC_{50} = 6.14 ± 1.13 nM). These data indicate that, like Kir6.2K338, Kir6.1R347 makes a crucial contribution to allosteric information transmission from SUR2A to the channel pore through inter-subunit salt bridge formation with SUR2A1318.

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Use of Resonance-Wavelength Grating Optical Biosensors to Detect Channel-Protein Interaction in Slack K_{Na} Channels

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Na^+-activated potassium (K_{Na}) channels encoded by the *Slack* and *Slick* genes contribute to neuronal adaptation during sustained stimulation and regulate the accuracy of timing of action potentials. Activation of protein kinase C (PKC) increases the amplitude of Slack-B currents and slows their rate of activation. Mutations in Slack channels which result in constitutive channel activation by mimicking phosphorylation cause malignant migrating partial seizures of infancy (MMPSI), a rare epileptic encephalopathy of infancy that combines pharmacoresistant seizures with severe developmental delay. Slack protein is known to interact with a variety of cytoplasmic signaling molecules. Using resonance wavelength grating optical biosensors (the SRU Biosciences BIND system), we have determined that direct pharmacological activation of Slack channels by bithionol produces a sustained decrease in mass distribution close to the plasma membrane, and that phosphorylation of Slack channels mimics this decrease in mass. The very C-terminal domain of Slack has been previously shown necessary for channel-protein interactions, and deletion of this region abolished the observed signal. To determine which proteins or signaling molecules are translocating from the plasma membrane upon channel activation, an RNAi screen against probable channel binding partners was performed, and the Protein Phosphatase 1 (PP1) targeting protein Phactr1 was found to be necessary for this decrease in mass. We hypothesize that activation of Slack by either bithionol or phosphorylation leads to the dissociation of Phactr1 with PP1 from the channel complex, allowing the Slack channel to remain in its phosphorylated and active state. Activation of PKC does not result in a decrease in mass in the human MMPSI mutants, possibly linking channel excitability to downstream signaling mechanisms which may result in developmental delay.

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Biogenesis of the Sodium-Activated Potassium Channel Slack-B is Controlled by the Dephosphorylation of N-Terminal Serines

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Slack-B channels are expressed predominantly in brainstem regions and the olfactory bulb. Using liquid-chromatography tandem mass spectrometry, we determined that two Ser residues (S34 and S44) close to the N-terminus of Slack-B are basally phosphorylated. To test the biological role of these sites we performed site-directed mutagenesis, creating mutations that mimic either phosphorylation (S->E) or dephosphorylation (S->A) at each or both site. We found that correlated with a 20-fold increase in whole-cell currents, levels of the nonphosphorylated mutant channel are greatly increased in cRNA-injected Xenopus oocytes as compared to wild type (WT) Slack. Next, comparing the rate of protein accumulation in oocytes injected with equal amounts of WT or mutant-encoding cRNA, we confirmed that the initial rate of protein accumulation is significantly increased for the nonphosphorylated channel. Additionally, the time constant for protein accumulation was much faster for nonphosphorylated channel, becoming saturated within two days. In contrast, levels of the WT channel accumulated with linear kinetics. We observed similar changes in Slack-B channel levels in HEK293 cells transiently transfected with bicistronic vectors carrying DsRed (transfected cell indicator) and WT or mutant Slack-Bl. To rule out the possibility that changes in current result from effects of the mutations on electrophysiologic parameters, we performed both macroscopic and single channel evaluation of each of the mutants. We found no significant differences among the WT and mutant Slack-B channels. Taken together, the results indicate that dephosphorylation of these sites in nascent peptide chains is a required step for permitting the translation of Slack mRNA to go to completion during channel synthesis. Our findings also suggest that regulation of the phosphorylation state of S34 and S44 may allow neurons to alter channel abundance rapidly in response to stimulation.

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Phospholipase C is Required for GαQ Protein-Coupled Receptor Mediated Task Channel Inhibition

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TWIK-related acid sensitive K^+ (TASK) channels belong to the K2P channel family and contribute significantly to the background conductance in various cell types, e.g. to $I_{K,SO}$ in cerebellar granule cells. It is known that stimulation of G_q-protein coupled receptors (G_qPCRs) causes strong and reversible inhibition of TASK channels. Yet, the underlying signaling cascade is still controversial: Both, the inhibition of TASK by $G\alpha_q$-protein via a direct molecular interaction, or, alternatively, by signals downstream of phospholipase C (PLC) activation have been proposed. PLC mediates the hydrolysis of phosphoinositide-4,5-bisphosphate ($PI(4,5)P_2$), thereby producing the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Here, we examine the requirement of PLC in G_qPCR-signaling for TASK-inhibition.

Whole cell patch clamp experiments were performed on CHO cells expressing TASK3 channels while PLC activity was modulated pharmacologically. PLC was activated through the application of the PLC-activator m-3M3FBS causing TASK3 current suppression. In contrast, G_q-mediated TASK3 inhibition by activation of muscarinic M1 receptor was abolished by the PLC inhibitor U73122.